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Decrease in macrophage antigen catabolism caused by ammonia and chloroquine is associated with inhibition of antigen presentation to T cells

(macrophage–lymphocyte interactions/T-cell immunity/lysosomotropic agents/antibacterial immunity)

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ABSTRACT **This paper describes the effects of two lysosomotropic compounds, ammonia and chloroquine, on the interaction of mononuclear phagocytes with immune T cells. The uptake and ingestion of *Listeria monocytogenes* by macrophages were not affected by the drugs; however, the macrophage catabolism of ^{125}I-labeled *Listeria* was reduced in a dose-dependent way. The macrophage presentation of *Listeria* to T cells, an *I*-region-dependent phenomenon, was also inhibited. The degree of inhibition of catabolism paralleled that of antigen presentation. The inhibition of antigen presentation took place if the macrophages were treated before and during *Listeria* uptake; minimal inhibition took place if the macrophages were treated 30 min after *Listeria* ingestion, at which time a significant amount of bacteria was already catabolized. Our previous studies had shown that the macrophage-associated antigen recognized by T cells became apparent 30–60 min after uptake of *Listeria*. We conclude that ammonia and chloroquine affected an intracellular handling step required for the expression of the immunogen relevant for T-cell recognition.**

Macrophages play a central role as antigen-presenting cells in the induction and expression of immune responses to most protein antigens (1, 2). It is widely held that an early event in the activation of T lymphocytes is their recognition of antigen present on the macrophage cell surface in association with gene products of the *I* region of the major histocompatibility locus (3). The precise mechanism, however, by which macrophages handle antigen in an immunologically relevant way remains to be elucidated. By using protein antigens under *I*-region control, several laboratories have shown that the immunogens appear to be small peptides that the macrophage selects for T cells to recognize (reviewed in ref. 4).

To study the mechanism of antigen presentation, we have used a system that involves the quantitation of the antigen-specific, *H-2I*-region-restricted binding of *Listeria monocytogenes*–immune T cells to macrophage monolayers (5). This system has the advantage that immunogenicity of a macrophage-bound antigen can be assessed in a short period of time, thereby permitting study of antigen-handling events—uptake, ingestion, and catabolism—in their relationship to presentation to immune T cells (6). We have recently shown that recognition of the *Listeria* immunogen by T cells takes place only after interiorization of the bacteria by the macrophage (6).

We now report that the selective inhibition of antigen catabolism by ammonia and chloroquine is associated with a corresponding inhibition of antigen presentation by macrophages. These results supply evidence for an intracellular pathway of antigen handling by macrophages relevant to T-cell recognition of antigen.

MATERIALS AND METHODS

The interaction of macrophages with *Listeria*–immune T cells as well as the handling of heat-killed *Listeria* by macrophages were studied in culture by methods described in detail in refs. 5 and 6.

Macrophages and T cells were isolated from peritoneal exudate cells of mice immunized with 10^4 live *L. monocytogenes* (5, 6). The macrophages (0.5–1.5 × 10^6 in 1 ml) were planted in 16-mm-diameter culture wells (Falcon, no. 3008), containing (if needed) a 15-mm coverslip, and portioned into three sets: one was cultured for 30 min at 37°C in RPMI-1640 medium with 5% fetal calf serum and antibiotics; the second and third sets were cultured with media containing either 10 mM NH_4Cl (Baker) or 0.1 mM chloroquine (Sigma).

The following assays were conducted in the continued presence of these agents.

Antigen uptake. Five million heat-killed ^{125}I-labeled *Listeria* organisms were spun onto the macrophage monolayer (5 min, 800 × *g*, 20°C) (the *Listeria* were labeled by the chloramine-T method and contained an average of 1 cpm per bacterium); the monolayer was washed and solubilized in 0.5% Triton-X, and the radioactivity in the supernatant was measured (7).

Antigen ingestion. Residual *Listeria* on the macrophage surface were counted after 20 min at 37°C (after their uptake); the macrophage monolayers were fixed in 1% paraformaldehyde, and the surface-bound *Listeria* were quantitated by immunofluorescence with rabbit anti-*Listeria* IgG and fluorescein-labeled goat anti-rabbit IgG (6). In previous experiments (6), we showed that the loss of surface-bound bacteria was attributed to ingestion and not to detachment from the membrane.

Antigen catabolism. We measured the release of 10% trichloroacetic acid-soluble radioactivity from the macrophage containing ^{125}I-labeled *Listeria* after 60 min of culture at 37°C (6).

I-A-*Positive macrophages.* The percentage of I-A-bearing macrophages was studied by immunofluorescence with monoclonal antibody clone 10-2.16 (8).

T cell–macrophage binding. A bioassay (5, 6) was used. A suspension of immune T cells was spun (50 × *g*, 5 min, 20°C) onto the macrophage monolayer and incubated 15 min at 37°C; the nonadherent lymphocytes were collected and assayed for immune T-cell reactivity in a second set of macrophage cultures. Immune T-cell reactivity was measured by either the production of a thymocyte mitogenic protein (9) or the development of macrophage tumoricidal activity (10). Both these assays give

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results identical to those of the standard T-cell proliferation test (5). Both assays are linear to the input of immune T cells and measure a macrophage–T cell interaction modulated by the *I* region (5–10). Results are reported as the percentage specific binding calculated from the decrease in reactivity of T cells recovered from macrophages incubated with *Listeria* compared to T cells recovered from macrophage not fed *Listeria*. The T-cell activities from macrophages not fed *Listeria* did not vary among the three experimental groups (i.e., untreated macrophages and macrophages treated with ammonia or chloroquine). Two protocols were used. In the "Before Antigen-Handling Protocol," macrophages were exposed to the drugs 30 min before and 30 min after the uptake of *Listeria*; in the "After Antigen-Handling Protocol," the macrophages were exposed to the drugs for 1 hr starting 30 min after the uptake of *Listeria*. In both cases, the agents were not present in the culture media during the 15-min T cell–macrophage binding reaction.

RESULTS AND DISCUSSION

We studied the effects of two lysosomotropic agents, ammonia and chloroquine, on the handling of heat-killed *L. monocytogenes* by macrophages and on the capacity of macrophages to present *Listeria* to immune T cells. Little or no inhibition of macrophage uptake and ingestion of *L. monocytogenes* was found with either drug (Table 1). During the 30- to 60-min exposure to the drugs, there was no gross redistribution or loss of membrane I-A from the macrophages. Two macrophage activities, however, were affected. The catabolism of ^{125}I-labeled *Listeria* was inhibited by approximately 40–60% in the presence of either of these agents as evidenced by the decrease in acid-soluble radioactivity found in culture fluids. Such inhibition was apparent throughout culture periods of 10 to 120 min during which the macrophage viability (as judged by trypan blue exclusion) remained unchanged. These results, in essence, confirm previous observations on the inhibitory effects of ammonia and chloroquine on protein degradation (11–13).

The second macrophage function inhibited was antigen presentation to immune T cells. When ammonia or chloroquine was present during a 30-min culture period prior to and following *Listeria* uptake (i.e., a total of 60 min), the interaction with T cells was markedly decreased in proportion to the amounts of drug (Table 1, Before Antigen Handling Protocol; Fig. 1). In contrast, when macrophages were exposed to *Listeria* for 30 min at 37°C in the absence of the agents and then incubated with them for 60 min at 37°C, only modest inhibition was observed (Table 1, After Antigen Handling Protocol). These results suggest that inhibitory effects of ammonia and chloroquine were exerted at the level of a macrophage antigen-handling event important in the generation rather than the maintenance (within the temporal framework studied) of macrophage-associated antigenicity important for T-cell recognition of antigen.

This analysis of macrophage antigen handling events in the context of antigen presentation function of macrophages reveals a striking association between the selective inhibition of antigen catabolism and the reduction in antigen presentation. Our recent study using the same methods (6) indicated that the optimal time for binding of T cells to macrophages was 30–60 min after *Listeria* uptake. At that time, the bulk of the bacteria was interiorized and found in intracellular vacuoles. Little or no binding of immune T cells took place at earlier times when the bacteria were surface bound or being phagocytized. Interestingly, T cells bound to paraformaldehyde-fixed macrophages that had taken up *Listeria*, implying that the binding substrate was available at the cell surface. The present results indicate that ammonia and chloroquine are affecting the intracellular handling step that resulted in the appearance of an immunogen for T cells.

Although the precise mechanism by which ammonia and chloroquine interfere with protein degradation is unclear, these agents are thought to exert their effects via disruption of normal lysosome function (12). Both agents increase lysosomal pH that then depresses the activity of acid hydrolases (14, 15). Chloroquine has also been shown to have a direct inhibitory effect on cathepsin B1 (12). Recently, ammonia has been shown to inhibit the fusion of phagosomes and lysosomes (16). It has also been suggested that ammonia and chloroquine may inhibit re-

Table 1. Inhibition of antigen presentation with NH_4Cl and chloroquine

Assay	Control, %	10 mM NH_4Cl Observed, %	10 mM NH_4Cl Δ,%	0.1 mM chloroquine Observed, %	0.1 mM chloroquine Δ,%
Antigen uptake*	15 ± 1	13 ± 2	13	15 ± 2	0
Antigen ingestion†	66 ± 2	63 ± 2	5	67 ± 6	−2
Antigen catabolism‡	29 ± 4	13 ± 3	**55**	14 ± 6	**52**
I-A expression§	54 ± 4	59 ± 2	−9	56 ± 4	−4
T cell–macrophage binding¶					
Before antigen handling	70 ± 7	26 ± 8	**63**	30 ± 8	**57**
After antigen handling	84 ± 8	70 ± 11	17	64 ± 10	24

* The results represent mean (±SEM) percentage of added ^{125}I-labeled *Listeria* bound to macrophages in two separate experiments; uptake in the absence of macrophages was <0.5%.

† Results represent the mean (±SEM) percentage decrease in surface-bound bacteria in two experiments. The control values for the number of *Listeria* per macrophage immediately after a 5-min exposure to *Listeria* was 30.4 ± 2.3 for untreated macrophages, 27.9 ± 2.5 for ammonia-treated macrophages, and 31 ± 8 for chloroquine-treated macrophages.

‡ The results represent the mean (±SEM) percentage release of trichloroacetic acid-soluble cpm after 1 hr of culture (three experiments).

§ Percentage of I-A-bearing macrophages.

¶ The results represent the percentage specific binding of *Listeria*–immune T cells to macrophages (mean of six experiments for Before Antigen Handling Protocol and four experiments for After Antigen Handling Protocol). Control values (T cells not adherent to macrophages not treated with *Listeria*) for T-cell activity ranged from 2176 ± 424 to 12,363 ± 731 with a mean of 7498 Δcpm in the mitogenic protein assay and ranged from 19.7 ± 0.9% to 36.8 ± 5.7% with a mean of 28.6% specific cytolysis in the induction of macrophage-mediated cytolysis assay. These values represent individual determinations of the mean ± SEM of duplicate control binding reactions.

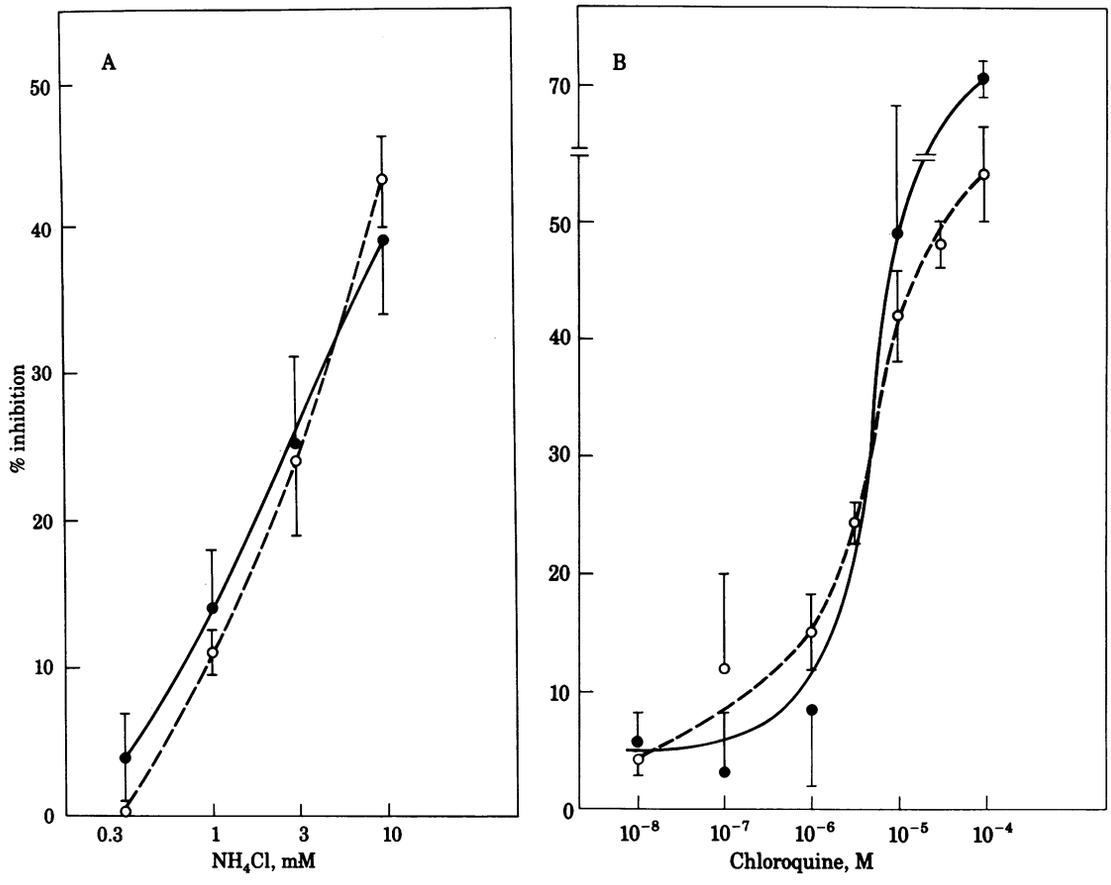

FIG. 1. Mean (±SEM; three experiments) percentage inhibition of antigen catabolism (○——○) and development of T cell–macrophage binding ability (●——●) is shown as a function of the final concentration of NH_4Cl (*A*) or chloroquine (*B*). The effects of these agents were analyzed by the Before Antigen Handling Protocol. Control values (no drug) for percentage of total ^{125}I-labeled *Listeria* released as acid-soluble material after a 60-min culture at 37°C were 22.1 ± 0.2, 19.7 ± 0.3, and 38.1 ± 0.1. Control values (no drug) for percentage specific T cell–macrophage binding were 85 ± 2, 56 ± 12, and 79 ± 4.

ceptor recycling (13). Regardless of the precise mechanism by which these agents exert their effects, they serve to identify a key intracellular pathway of antigen handling by macrophages.

It is interesting to note that the generation of ammonia by some intracellular pathogens (e.g., *Mycobacterium tuberculosis*) (16) may represent a means to subvert immunological reactivity by reducing the obligate antigen presentation function of the macrophage. Also, ammonia generated as a product of amino acid metabolism may represent a physiological control mechanism of protein degradation (17). We speculate that anergy associated with hyperammonemia in certain clinical settings (e.g., acute hepatic failure and starvation) may be mediated by disruption of lysosomal function and thus antigen presentation.

This and other studies (6, 18) support the idea that macrophage presentation of immunogens to T cells requires intracellular handling of the antigen molecule. It is possible that, after lysosomal digestion, antigen fragments are transferred to accessible sites at the cell surface or are released to the extracellular milieu (19–21) where they may interact with *I*-region products and with specific T cells. Our observation may explain the observations that the antigenic determinants of protein antigens recognized by T cells are represented by denatured portions of the proteins (22–27) or by small sequences of amino acids (28–30). Such changes in the protein would be expected to take place after partial lysosomal digestion. The intracellular handling step described herein, however, may not take place in all situations or with all antigens. We have selected for our studies a complex antigen structure—bacterium—for which one may expect an obligatory initial catabolic processing. It is possible that such an intracellular step may not be required, or may be bypassed, with small peptide antigens such as those used in some of the bioassays for T-cell function. It should also be noted that presentation of globular protein to B cells may involve nonprocessed molecules (1). Finally, we speculate that the degradation of bacterial antigen by macrophages may have a survival value by increasing the number of structural moieties that can serve as immunogens, making it less likely that a nonresponder status with respect to *I*-region function will be generated.

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1. Unanue, E. R. (1981) *Adv. Immunol.* **31**, 1–136.
2. Möller, G., ed. (1978) *Immunol. Rev.* **40**, 1–236.
3. Shevach, E. M. & Rosenthal, A. S. (1973) *J. Exp. Med.* **138**, 1213–1218.
4. Rosenthal, A. S. (1980) *N. Engl. J. Med.* **303**, 1153–1164.
5. Ziegler, H. K. & Unanue, E. R. (1979) *J. Exp. Med.* **150**, 1143–1160.
6. Ziegler, H. K. & Unanue, E. R. (1981) *J. Immunol.* **127**, 1869–1875.
7. Weinberg, D. S. & Unanue, E. R. (1981) *J. Immunol.* **126**, 794–802.
8. Beller, D. I., Kiely, J.-M. & Unanue, E. R. (1980) *J. Immunol.* **124**, 1426–1436.

9. Farr, A. G., Kiely, J.-M. & Unanue, E. R. (1979) *J. Immunol.* **122,** 2395–2404.
10. Farr, A. G., Wechter, W. J., Kiely, J.-M. & Unanue, E. R. (1979) *J. Immunol.* **122,** 2405–2415.
11. Seglen, P. O., Grinde, B. & Solheim, A. E. (1979) *Eur. J. Biochem.* **95,** 212–225.
12. de Duve, C., de Barsy, T., Poole, B., Trovet, A., Tulkens, P. & Van Hoof, F. (1974) *Biochem. Pharmacol.* **23,** 2495–2531.
13. Tietze, C., Schlesinger, P. & Stahl, P. (1980) *Biochem. Biophys. Res. Commun.* **93,** 1–8.
14. Ohkuma, S. & Poole, B. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 3327–3331.
15. Gersow, M. J., D'Arcy Hart, P. & Young, M. R. (1981) *J. Cell Biol.* **89,** 645–652.
16. Gordon, A. H., D'Arcy Hart, P. & Young, M. R. (1980) *Nature (London)* **286,** 79.
17. Holzer, H. & Heinrich, P. C. (1980) *Annu. Rev. Biochem.* **49,** 63–91.
18. Ellner, J. J., Lipsky, P. E. & Rosenthal, A. S. (1977) *J. Immunol.* **118,** 2053–2068.
19. Calderon, J. & Unanue, E. R. (1974) *J. Immunol.* **112,** 1804–1816.
20. Erb, P., Meier, B. & Feldmann, M. (1979) *J. Immunol.* **122,** 1916–1928.
21. Lonai, P., Puri, J. & Hämmerling, G. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 549–553.
22. Gell, P. H. G. & Benacerraf, B. (1959) *Immunology* **2,** 64–83.
23. Ishizaka, K., Kishimoto, T., Delesperse, G. & King, T. P. (1974) *J. Immunol.* **113,** 70–74.
24. Shirrmacher, V. & Wigzell, H. (1974) *J. Immunol.* **113,** 1635–1643.
25. Chestnut, R. W., Endres, R. O. & Grey, H. M. (1980) *Clin. Immunol. Immunopathol.* **15,** 396–408.
26. Thommpson, K., Harris, M., Benjamin, E., Mitchell, G. & Noble, M. (1972) *Nature (London) New Biol.* **230,** 20–24.
27. Parish, C. R. & Liew, F. Y. (1972) *J. Exp. Med.* **135,** 298–312.
28. Barcinski, M. A. & Rosenthal, A. S. (1977) *J. Exp. Med.* **134,** 1529–1540.
29. Thomas, D. W., Meltz, S. K. & Wilner, G. D. (1979) *J. Immunol.* **123,** 759–764.
30. Thomas, D. W., Meltz, S. K. & Wilner, G. D. (1979) *J. Immunol.* **123,** 1299–1302.